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Fatty acid composition in native bees: Associations with thermal and feeding ecology

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ABSTRACT

Fatty acid (FA) composition of lipids plays a crucial role in the functioning of lipid-containing structures in organisms and may be affected by the temperature an organism experiences, as well as its diet. We compared FA composition among four bee genera: *Andrena, Bombus, Megachile,* and *Osmia* which differ in their thermal ecology and diet. Fatty acid methyl esters (FAME) were prepared by direct transesterification with KOH and analyzed using gas-liquid chromatography with a flame ionization detector. Sixteen total FAs ranging in chain length from eight to 22 carbon atoms were identified. Linear discriminant analysis separated the bees based on their FA composition. *Andrena* was characterized by relatively high concentrations of polyunsaturated FAs, *Bombus* by high monounsaturated FAs and Megachilids (*Megachile* and *Osmia*) by relatively high amounts of saturated FAs. These differences in FA composition may in part be explained by variation in the diets of these bees. Because tongue (proboscis) length may be used as a proxy for the types of flowers bees may visit for nectar and pollen, we compared FA composition among *Bombus* that differed in proboscis length (but have similar thermal ecology). A clear separation in FA composition within *Bombus* with varying proboscis lengths was found using linear discriminant analysis. Further, comparing the relationship between each genus by cluster analysis revealed aggregations by genus that were not completely separated, suggesting potential overlap in dietary acquisition of FAs.

1. Introduction

Eukaryotic cells devote substantial resources (~5% of their genes) to producing and maintaining lipid assemblages that provide key evolutionary advantages (Sud et al., 2007; van Meer et al., 2008; Vance and Vance, 1985). Lipids serve as key energy reserves (Arrese and Soulages, 2010; Hahn and Denlinger, 2007), are fundamental components of cell membranes (Hochachka and Somero, 2002), and play key roles in neural signaling processes (reviewed in van Meer et al., 2008). Variation in the functional demands and diet availability of lipids can lead to consistent differentiation of lipid profiles among organisms and habitats (Castell et al., 1972; Hanson et al., 1985). As such, lipid profiles can be used to differentiate among certain taxa (Arts and Wainmann, 2012; Brett and Müller-Navarra, 1997; Torres-Ruiz et al., 2007).

Temperature and diet are the two primary factors that can drive differentiation of lipid profiles among organisms (Hanson et al., 1985; Hazel, 1995; Hochachka and Somero, 2002) through effects on physicochemical properties of lipid constituents. The functioning of lipids, and hence lipid-containing structures, depends strongly on the physicochemical properties of lipid constituents (Hochachka and Somero, 2002), particularly fluidity. Because lipids are key components of biological membranes, changes in lipid composition can alter membrane fluidity, which can, among other things, disrupt ion balance and the function of lipid-derived second messengers (reviewed in Hazel, 1995). Additionally, given that lipids can only be metabolized when fluid (Frank, 1992; Holmstrup et al., 2007; Kostal and Simek, 1998; Ohtsu et al., 1993; Ruf and Arnold, 2008), changes in lipid fluidity can also alter metabolism and energy production.

Fluidity of lipids is determined by various factors including the ratio of unsaturated to saturated fatty acids (UFA:SFA) that constitute the lipid (Hochachka and Somero, 2002). The ratio of UFA to SFA is primarily determined by the temperatures an organism experiences (Hazel, 1995; e.g. Marr and Ingraham, 1962) and by its diet (Barlow, 1966; Frank, 1992). Whereas UFAs provide less energy when metabolized but maintain fluidity at low temperatures, SFAs are less fluid at low temperatures but provide relatively more energy when metabolized. Therefore, based on the homeoviscous adaptation (HVA) hypothesis, organisms experiencing colder temperatures should have

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higher UFA:SFA (to maintain fluidity and functionality of lipid containing structures), whereas organisms experiencing warmer conditions should favor SFAs for their increased energy yield (although the quantity may vary with tissue under study). The basic predictions of the HVA hypothesis (Sinensky, 1974) have been tested and appear to hold for diverse organisms (Anderson et al., 1981; Carey and Hazel, 1989; Fudge et al., 1998; reviewed in Hazel, 1995; Holmstrup et al., 2007; Raynard and Cossins, 1991) including insects (Atapour et al., 2007; Barlow, 1964; Fast, 1966; van Dooremalen and Ellers, 2010). Insects often show strong shifts in fatty acid (FA) composition in response to temperature (e.g. Hahn and Denlinger, 2011; see Hazel, 1995; Kostal and Simek, 1998), likely in part because, as small ectotherms they have limited scope for decoupling body temperature from environmental temperature and therefore must cope physiologically with environmental temperature fluctuations.

Changes in FA composition that facilitate tolerance of diverse environmental temperatures arise from three main processes: de novo synthesis of FAs, assimilation of FAs from the diet, and modification of existing FAs. Although insects, like other organisms, can synthesize many FAs de novo, polyunsaturated fatty acids (PUFAs) must be obtained from the diet (reviewed in Stanley-Samuelson et al., 1988). Consequently, the amount of these essential FAs in the diet will influence FA distribution in organism tissues (Barlow, 1966; Frank, 1992; Frank et al., 2008). Furthermore, the composition and concentrations of FAs in the diet that are precursors for the synthesis of other FAs may also alter the concentrations of FAs that are synthesized de novo, ultimately affecting insect FA composition. Thermal and feeding ecology therefore both independently and interactively, may be strong determinants of and provide useful information on FA biochemistry in insects and the mechanistic basis for their adaptations to predicted changes in environmental conditions (Hartmann et al., 2013).

Native bees vary widely in body size (Michener et al., 1994) and associated thermal ecology (Bishop and Armbruster, 1999; Stone and Willmer, 1989), as well as in diet (Michener et al., 1994; Willmer, 2011), providing an excellent group for comparative studies of FA composition. Body temperatures of small bees likely track changes in environmental temperatures, whereas larger bees and those that live in social aggregations can regulate body temperatures physiologically (via regulation of loss of metabolic heat; Heinrich, 1974a, 1974b), and behaviorally (Heinrich, 1975; Heinrich and Esch, 1994; Kronenberg and Heller, 1982). For example, eusocial bees nesting together can maintain relatively constant and warm hive temperatures (Engels et al., 1995; Heinrich, 1975; Kronenberg and Heller, 1982), unlike solitary bees (see Heinrich, 1993). Bee morphology and behavior also influence the types of flowers bees visit and their ability to collect pollen and nectar (Brian, 1947; Michener et al., 1994; Willmer, 2011) with possible effects on dietary FAs and hence, on tissue FA composition.

We compared FA composition among four bee genera (*Andrena*, *Bombus*, *Megachile* and *Osmia*) that differ in their thermal ecology and diet. The smallest bees in this study, *Andrena* (mean wet mass = 0.06 g) and *Osmia* (mean wet mass = 0.05 g), may be less likely to retain metabolic heat compared to *Bombus* (mean wet mass = 0.25 g) and *Megachile* (mean wet mass = 0.12 g) given their greater surface area to volume ratio (Stone and Willmer, 1989) and given the higher pilosity of *Bombus* (and to some extent, *Megachile*). We, therefore, expected higher UFA: SFA in *Andrena* and *Osmia* relative to *Bombus* and *Megachile* collected in similar environments. *Bombus* are heterothermic and eusocial and hence benefit from elevated and constant body temperatures when foraging and return to nests with regulated temperatures (Heinrich, 1993, 1975). Lipid profiles in *Bombus* may therefore be less influenced by environmental temperatures compared to the other three genera.

The four bee genera in this study also vary in their feeding ecology. Differences in proboscis length and body size in part determine from which flowers bees can successfully extract nectar and pollen (Willmer, 2011). Variation among plants in lipid composition of pollen (and to a lesser extent nectar, Willmer, 2011) could result in differences in lipid composition of bee diets. This variation in lipids in the diet could subsequently lead to differences in lipid profiles of bee tissues.

We describe a protocol adapted for FA composition analysis in bees using gas-liquid chromatography with a flame ionization detector (GC-FID). We then measure FA composition of 73 bees from four bee genera: *Andrena, Bombus, Megachile* and *Osmia*. We describe clear differences in FA composition among genera, likely related to thermal ecology and diet. We further show that FA profiles differ with proboscis length in bumble bees. Using cluster analysis, we also show that genus aggregations are not completely separated, suggesting potential overlap in dietary acquisition of FAs.

2. Methods

2.1. Field collection of samples

We collected bees near Phelps Lake in Grand Teton National Park (N 43° 30.220′, W 110° 48.327′, 2060 m asl; June to August 2012) in an open meadow with abundant flowers supporting a diverse bee community. Bees were euthanized in cyanide within 30 s of capture and subsequently transported on ice to the University of Wyoming (UW)-National Park Service (NPS) Research Station. Bee body mass was measured in the field within 24 h of capture (± 1 mg, Acculab PP2060D) and then re-measured within eight days of capture when samples were transported to the lab (± 0.1 mg, Acculab ALC-210.4, NY, USA). We identified bees to genus and bumble bees to species (all workers, Koch et al., 2012; Michener et al., 1994). All analyses were performed on female bees for *Andrena*, *Bombus* and *Megachile*. However, given that none of the analyses showed variation among sexes, male and female data were combined for analyses in *Osmia*. We then cleaned off any pollen particles present on the body, and then stored the bees at −20 °C until performing FA composition analyses.

2.2. Extraction and methylation of fatty acids

All reagents were analytical grade and glassware and utensils were washed with Liquinox (Alconox, Inc.) followed by hexane to eliminate organic residue prior to usage. Using lab-reared *Bombus impatiens*, we first compared three approaches to fatty acid methyl ester (FAME) preparation for FA analysis in bees using gas-liquid chromatography (GLC): i. methylation of directly saponified FAs, ii. direct transesterification using methanolic HCl, iii. direct transesterification using methanolic KOH (KOH approach). Triacylglycerol (tritridecanoate, C 13:0, 1 mg) was used as an internal standard (IS) for all methods. Fourteen worker *B. impatiens* collected from a lab-reared hive were lyophilized (Freezone 4.5, Labconco, Kansas City, USA) and homogenized (Tekmar, Telex no: 21-4221, Vernon, BC, Canada) together and subsamples from this homogenate were used for FA analysis to compare the three methods.

For methylation of directly saponified tissue, we followed procedures as described by Lake et al. (2006). Briefly, in the homogenized sample placed in 16 × 125 mm glass tubes with teflon-lined screw caps, we added 4 mL of ethanol and 1 mL of 33% (wt/vol) KOH in DI water followed by heating at 80 °C for 60 min, vortexing every 5 to 10 min. We next added 1 mL of 12 M HCl and 3 mL high purity hexane, vortexed and centrifuged at 2600 rpm for 3 min (the same conditions apply hereafter unless otherwise mentioned; Beckman TJ-6/TJ-6R centrifuge, MN, USA). The supernatant layer containing hexane and FAs was transferred to a clean tube with IS. We then dried the samples (evaporation of hexane) using N_2 gas at 50 °C, added 4 mL 0.545 M methanolic HCl, and incubated at 80 °C for 45 min, vortexing every 5 min. We next added 2 mL of DI water and 1 mL high purity hexane, centrifuged for 3 min and transferred the supernatant, the hexane layer containing FAs, to gas liquid chromatography (GLC) auto-sampler vials (2 mL, 13 × 32 mm, Agilent Technologies, USA) containing a bed of Na_2SO_4, and stored at −20 °C until run in the GC.

We followed Weston et al. (2008) to perform direct transesterification of bee tissue using methanolic HCl. Briefly, homogenized bee samples were placed in 16 × 125 mm glass tubes with teflon-lined screw caps. We added 2 mL each of chloroform (Acros Organics, HPLC) and an aqueous solution containing 1 M KCl and 0.15 N HCl to the tube, vortexed for 30 s (Vortex Genie 2, Scientific Industries; NY, USA) and centrifuged at 2600 rpm for 3 min. The centrifugation led to the separation of phases with an upper layer of debris and a lower layer containing chloroform and extracted FAs. The lower layer with chloroform was transferred to a clean tube containing 1 mg of internal standard (IS, tridecanoin/C 13:0; prep, Inc., MN, USA). We re-extracted the residue in the original tube by adding 4 mL chloroform, centrifuging as above and transferring the chloroform layer to the same tube with the first extract. After drying the chloroform using N_2 gas (Meyer N-EVAP Analytical Evaporator, Organomation, Berlin, USA), we added 6 mL of methanolic HCl (0.545 N HCl in methanol, Acros Organics, HPLC, 99.9%), vortexed for 3–5 s and heated at 85 °C (Select Heatblock, VWR, Scientific Products, NJ, USA) for 60 min, vortexing every 5–10 min. Next, we added 2 mL each of deionized (DI) water and hexane (> 98%, HPLC plus, Sigma Aldrich), vortexed, and then centrifuged for 5 min. This allowed for phase separation with the hexane layer containing FAs as the supernatant and the other tissues in the bottom. The top layer was carefully transferred to GLC auto-sampler vials containing a bed of Na_2SO_4, and vials were stored at −20 °C until run in the GC.

We performed direct transesterification with KOH as described by Murrieta et al. (2003) with some modifications. We added 6 mL methanol (for efficient homogenization of the samples) in the tubes with IS and lyophilized bees. We next homogenized the sample, added 1 mL 1.4 M KOH in methanol to make a final concentration of 0.2 M KOH in methanol and incubated at 65 °C for 60 min, vortexing every 5 min. We then added 2 mL each of DI water and hexane, vortexed and centrifuged at 2800 rpm for 5 min, which led to the phase separation, with the supernatant containing hexane and methylated FAs. The supernatant layer was transferred to GLC auto-sampler vials with a bed of NA_2SO_4 and vials were stored at −20 °C until run in the GC.

Among the three methods tested, direct transesterification with KOH was best suited for application in bees as small as 0.009 g wet mass (0.004 g dry mass). This approach has previously been used successfully with animal tissue (Murrieta et al., 2003) and requires minimal handling of samples, reducing the possibility of losing tissue, particularly when working with small samples. This approach was also faster and provided higher resolution compared to direct saponification: auto integration detected more peaks under similar conditions, and the area under the curve for peaks detected by both methods (methylation of directly saponified FAs and KOH approach) was significantly higher using the KOH approach (paired *t*-test, $t_7 = -3.2$, $P = 0.015$). We couldn't reliably obtain clear GC traces using the direct transesterification with methanolic HCl approach and hence data are not shown for this method. We therefore adopted the KOH approach to trans-esterify and determine FA composition of homogenized body tissues of *Andrena* (12 females), *Bombus* (26 females), *Megachile* (12 females), and *Osmia* (14 females, 9 males).

2.3. Gas chromatography

Fatty acid methyl esters were extracted in hexane and 1 μL of the extraction injected into the GC (Agilent Technologies 6890 N), equipped with a flame ionization detector and a 60 m × 0.25 mm fused silica capillary column (0.25 μm film thickness; DB-23, Agilent Technologies). Oven temperature was maintained at 75 °C for 1 min and then increased to 170 °C at a rate of 6.5 °C/min. Oven temperature was held at 170 °C for 27 min, then increased to 215 °C at 10 °C/min and held for 30 min. Finally, the temperature was raised to 230 °C over a period of < 1 min and then held for 3 min. Hydrogen gas was used as carrier with a split ratio of 50:1 and a split flow of 35.5 mL/min. FA peaks were recorded and integrated using GC ChemStation software (Agilent Technologies, version A.09.03). Individual FAMEs were identified by co-elution with known FAME standards spiked into samples (Nu-chek prep, Inc., MN, USA). Mass of identified FAMEs was calculated by comparing the area under the peak for a particular FAME to the area under the peak for known amounts of internal standard (tridecanoate).

2.4. Statistical analyses

All statistical analyses were performed using R (R Core Team, 2016). Welch two-sample *t*-tests were used to compare variables across sexes. Single factor analysis of variance (ANOVA) was used to assess the distribution and profile of FAs across genera and among *Bombus* species classified by proboscis length as short-tongued (S-T), medium-tongued (M-T) or long-tongued (L-T) following Koch et al. (2012) and Williams et al. (2014). *Bombus appositus* (5) and *B. flavifrons* (13) were grouped as L-T bees, *B. bifarius* (3) and *B. mixtus* (1) as M-T bees and *B. insularis* (1), *B. rufocinctus* (2), and *B. fernaldae* (1) as S-T bees. FAs were expressed as mg of individual FA per 100 mg of total identified FA, essentially a normalized mass percentage. These data were then logit transformed prior to analyses. Differences among FA components were assessed with Tukey's HSD. To reduce dimensionality and facilitate comparison of FA composition across bee genera, we performed linear discriminant analysis (Venables and Ripley, 2013) on the logit-transformed (Warton and Hui, 2010) percentage FA composition. We also performed hierarchical cluster analysis ("stats" package, "hclust" function, R Core Team, 2016) to detect clustering of bee genera sharing common FA composition. For cluster analysis, we employed Ward's method using Euclidean distance.

3. Results

3.1. Fatty acid distribution

The percent composition by mass of all identified FAs averaged among individuals for all bees and for each genus are presented in Table 1. For example, oleic acid (C18:1) was found in 71 out of 73 bees and made up on average 35% of the FAs identified in each bee. We identified a total of 16 FAs across all bee samples. Long-chain FAs (LCFAs) with chain length ranging from 14 to 20 carbon atoms and very long-chain FAs with chain lengths up to 22 carbon atoms were most common, accounting for over 99% of total identified FA across all bees. The remaining FAs were medium-chain FAs (MCFAs), with chain lengths ranging from 8 to 12 carbon atoms; MCFAs were completely absent in *Osmia*, and rare in the other three genera (Table 1). Monounsaturated fatty acids (MUFA) accounted for over 50% of total FA mass for all four genera (Fig. 1) and were significantly different among genera (ANOVA, $F_{3,\ 264} = 75.4$, $P < 0.001$). MUFAs were in highest proportions in *Bombus* spp. followed by *Osmia* spp. The proportion of MUFAs was significantly different between each pair of genera (TukeyHSD, all $P < 0.001$) except between *Andrena* and *Megachile* (TukeyHSD, $P = 0.934$). The proportion of PUFAs also varied significantly among genera ($F_{3,\ 171} = 15.6$, $P < 0.001$), with the lowest proportion in *Bombus* (~15.7% of total FAs identified) and the highest proportion in *Andrena* (~37%) and was significantly different among genus pairs (TukeyHSD, all $P < 0.05$) except for *Megachile* and *Osmia* (TukeyHSD, $P = 0.800$). These related genera within the family Megachilidae both had a higher proportion of SFAs relative to *Andrena* and *Bombus* (TukeyHSD $P < 0.02$ for all combinations except for *Megachile-Andrena*, $P = 0.298$). Overall, the proportion of MUFAs was highest in *Bombus*, that of PUFAs was highest in *Andrena*, and that of SFAs was highest in the Megachilids. The percentages of MUFAs, PUFAs, and SFAs were similar between male and female *Osmia* ($t_{63} = -0.2$, $P = 0.806$, $t_{34} = 0.4$, $P = 0.698$, and $t_{36} = -0.03$, $P = 0.979$, respectively) and between *Bombus* with varying proboscis length (all $P > 0.111$).

Because tissue mass directly affects the mass of total FAME

Table 1
The percent composition by mass of all identified FAs averaged among individuals for all bees and for each genus. Values are mean ± sd, with numbers in parentheses indicating the number of individuals in which the FA was present. Data are sorted based on overall FA prevalence with the FAs present in the highest number of individuals listed first. Because percentages were first calculated for individual bees and all bees did not have all FAs, the columns won't necessarily sum to 100%.

FA	All bees combined (N = 73)	*Andrena* (N = 12)	*Bombus* (N = 26)	*Megachile* (N = 12)	*Osmia* (N = 23)
C 16:0	10.4 ± 3.9 (73)	9.5 ± 3.1 (12)	8.4 ± 3.4 (26)	11.1 ± 2.6 (12)	12.8 ± 4.2 (23)
C 18:3	14.6 ± 6.4 (73)	22.4 ± 5.4 (12)	10.0 ± 3.5 (26)	15.1 ± 4.4 (12)	15.6 ± 6.2 (23)
C 18:1	35.0 ± 8.0 (71)	26.0 ± 7.5 (10)	37.0 ± 8.2 (26)	34.8 ± 3.8 (12)	36.7 ± 7.1 (23)
C 20:1	13.6 ± 6.8 (70)	6.9 ± 3.5 (9)	15.8 ± 4.6 (26)	10.4 ± 6.6 (12)	15.4 ± 7.9 (23)
C 16:1	11.8 ± 9.8 (67)	18.7 ± 14.8 (12)	14.2 ± 9.3 (26)	6.1 ± 2.3 (11)	7.2 ± 4.3 (18)
C 18:0	5. 2 ± 2.4 (57)	4.2 ± 1.9 (4)	4.1 ± 1.6 (25)	6.7 ± 2.6 (12)	6.0 ± 2.7 (16)
C 20:5	4.6 ± 2.7 (52)	2.8 ± 1.6 (6)	4.9 ± 2.2 (25)	3.3 ± 1.0 (10)	6.6 ± 3.8 (11)
C 18:2	7.6 ± 5.4 (50)	14.2 ± 3.8 (11)	2.0 ± 0.8 (14)	9.5 ± 4.1 (12)	6.2 ± 3.1 (13)
C 14:0	1.7 ± 1.1 (39)	2.0 ± 1.0 (6)	1.1 ± 0.5 (24)	3.1 ± 0.7 (7)	3.6 ± 0.5 (2)
C 22:1	3.4 ± 2.8 (31)	–	1.8 ± 0.8 (16)	3.2 ± 1.8 (5)	6.2 ± 3.2 (10)
C 14:1	3.7 ± 3.1 (29)	6.7 ± 4.3 (8)	2.6 ± 1.5 (21)	–	–
C 12:0	0.6 ± 0.5 (23)	1.3 ± 0.6 (3)	0.5 ± 0.4 (19)	0.9 (1)	–
C 20:0	0.5 ± 0.5 (20)	0.4 (1)	0.4 ± 0.2 (17)	1.7 ± 0.0 (2)	–
C 21:0	0.9 ± 1.0 (20)	2.9 ± 1.2 (3)	0.6 ± 0.5 (17)	–	–
C 8:0	1.8 ± 1.7 (5)	1.4 (1)	0.3 ± 0.1 (2)	3.6 ± 0.3 (2)	–
C 10:0	0.5 ± 0.7 (4)	1.5 (1)	0.2 ± 0.1 (3)	–	–

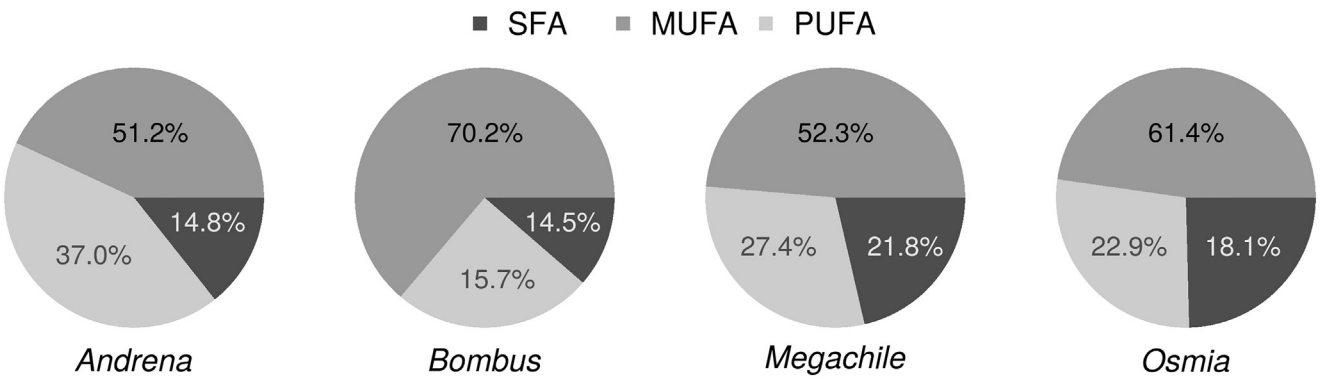

Fig. 1. Variation in major fatty acid types among bee genera. Pie charts show the mean percentage of monounsaturated (MUFA), polyunsaturated (PUFA) and saturated fatty acids (SFA) for each bee genus.

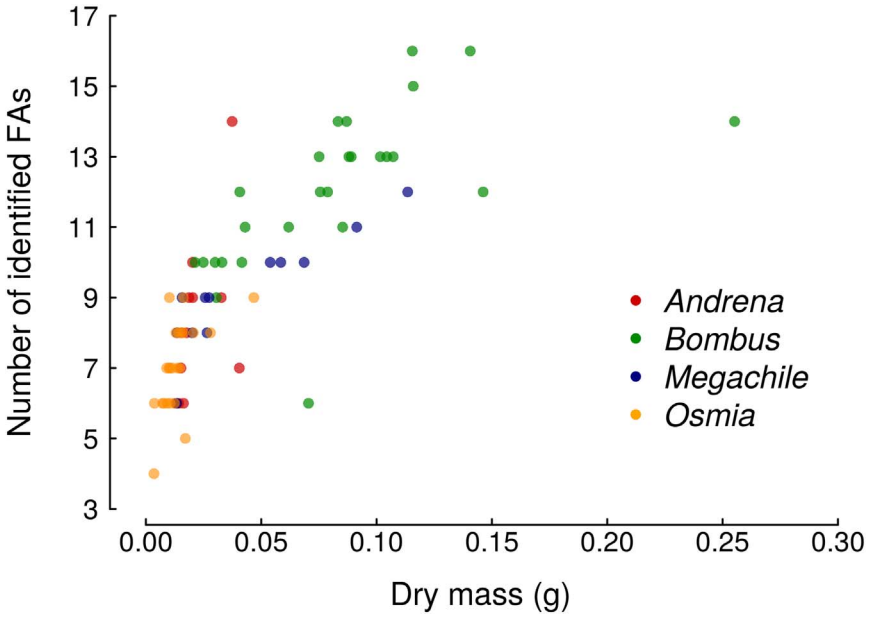

Fig. 2. Effect of dry mass on total number of fatty acids identified. The number of identified FAs increased with increasing dry mass for *Bombus* (green points) and *Megachile* (blue points) but did not differ with dry mass for *Andrena* (red points) or *Osmia* (orange points, see text for details and statistics). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extracted (e.g. insect samples are often pooled to increase the tissue mass for more consistent peak integration and identification), we evaluated the total number of FAs identified relative to the dry mass of bees. However, because tissue mass varied by genus, we excluded dry mass from our models for comparing all other variables. Overall, the number of identified FAs increased with bee dry mass within the genus (dry mass: $F_{1,65} = 151.9$, $P < 0.001$; genus: $F_{3,65} = 7.7$, $P < 0.001$; dry mass, genus interaction: $F_{3,65} = 2.3$, $P = 0.081$, Fig. 2). Bumble

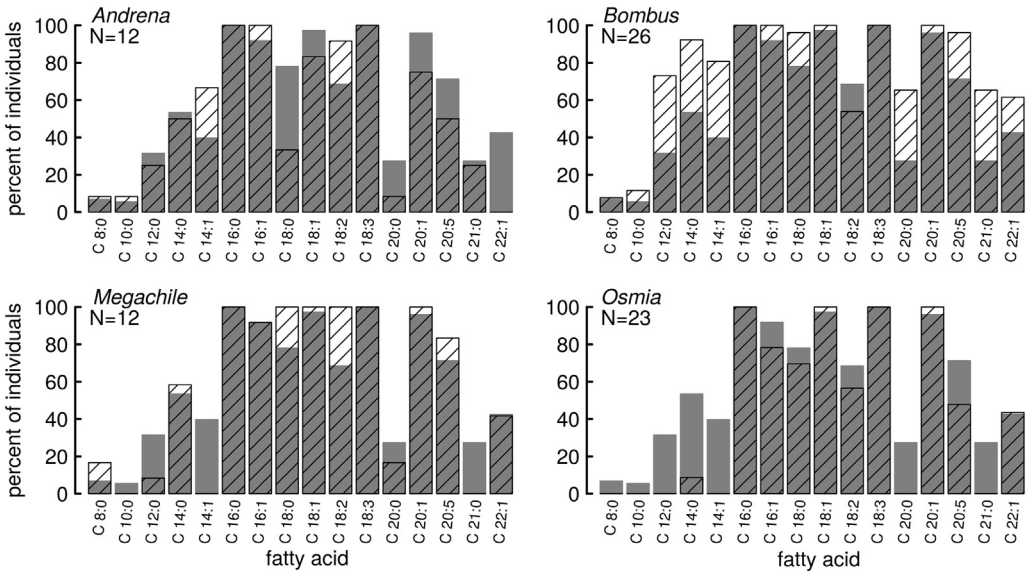

Fig. 3. Prevalence of fatty acids across four bee genera. Gray shaded bars represent mean prevalence for all genera studied (in the background) and overlaid hatched bars show the prevalence for a given genus.

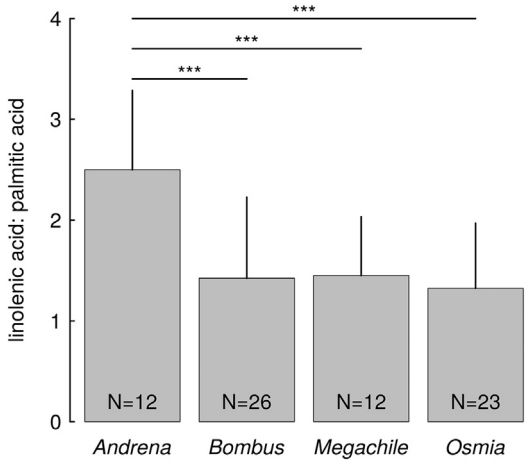

Fig. 4. The ratio of linolenic to palmitic acid varied significantly among bee genera. Bars represent mean + sd for the individuals in a genus. Significant pairwise comparisons are shown by black bars (***$P \leq 0.001$; see text for statistical details).

bees had the most diverse complement of FAs, with the other genera lacking one or more of the FAs found in *Bombus* (Table 1). Even within *Bombus*, the number of identified FAs was significantly higher for larger bees within a class (S-T, M-T, or L-T; dry mass: $F_{1,20} = 15.3$, $P < 0.001$; genus: $F_{2,20} = 1.4$, $P = 0.275$; dry mass, genus interaction: $F_{2,20} = 2.8$, $P = 0.083$). *Andrena* was marked by the complete absence of C 22:1, which was detected (albeit at low levels) in all three of the other genera, in 42% of all individuals. Megachilids (*Megachile* and *Osmia*), on the other hand, were marked by complete absence of C 10:0, C 14:1 and C 21:0 where *Osmia* additionally lacked C 8:0, C 12:0 and C 20:0 (Fig. 3). The number of FAs identified was similar in male and female *Osmia* ($t_{21} = 0.5$, $P = 0.623$).

C 18:3 (linolenic acid) and C 16:0 (palmitic acid) were detected in all bees (Table 1). The proportions of these ubiquitous FAs varied among genera ($F_{3,69} = 9.9$, $P < 0.001$), being significantly higher for *Andrena* compared to the other three genera (TukeyHSD, all $P < 0.005$), which were not different (TukeyHSD, all $P > 0.774$; Figs. 3, 4). The ratio of linolenic to palmitic acid was independent of sex ($t_{16} = 0.2$, $P = 0.9$); hence, data for both male and female *Osmia* were combined for comparison among genera.

Because certain PUFAs including C 18:3 are essential for insects, the composition and amounts of dietary FAs may determine the FA composition in insect tissues. We therefore compared the ratio of linolenic to palmitic acid among three groups of *Bombus* with similar thermal ecology, but different feeding ecologies, grouped based on their proboscis lengths. The ratio of linolenic to palmitic acid was markedly lower for L-T bees compared to S-T and M-T bees (TukeyHSD, both $P < 0.05$) with S-T- and M-T bees similar (TukeyHSD, $P = 0.963$).

Oleic acid (C 18:1) made up the greatest fraction in individuals (Fig. 5) and varied significantly across genera ($F_{3,\ 67} = 7.3$, $P < 0.001$). *Andrena* had a significantly lower fraction (26.0 ± 7.5%) of C18:1 (all $P < 0.05$) than did *Bombus* (37.0 ± 8.2%), *Megachile* (34.8 ± 3.8%) and *Osmia* (36.7 ± 7.1%), all of which were similar (TukeyHSD, all $P > 0.817$). We found no effect of sex in *Osmia* ($t_{21} = -0.9$, $P = 0.3$) or of proboscis length in *Bombus* ($F_{2,23} = 1.4$, $P = 0.256$) on the fraction of C 18:1 detected. On average, UFA: SFA, although not different between sexes ($t_{107} = -0.1$, $P = 0.923$), varied significantly among genera ($F_{3,68} = 127.4$, $P < 0.001$; TukeyHSD, all $P < 0.001$, Fig. 6).

3.2. Linear discriminant analysis

Using linear discriminant analysis, bee genera were well-separated where the first, second and third discriminant functions accounted for 55.6%, 32.8% and 11.6% of the discriminative power, respectively. *Andrena* was separated from other 3 genera along the first linear discriminant (LD1) with higher percentage of C 18:2 and C 10:0 (Fig. 7 a & b, Table 1). *Bombus* was in turn separated from the Megachilids by the second discriminant (LD2) with presence of C 14:1 and C 21:0 and relatively high C 18:1 and C 20:1 and low C 16:0. Finally, the Megachilids were well-separated from each other by the third discriminant function (LD3, indicated by point size in Fig. 7). *Megachile* was distinguished by the presence of high C 8:0 and C 18:2 and *Osmia* was marked by relatively high levels of C 16:0 and C 22:1.

The observable differences in FA composition among the genera may reflect variation in bee diets. We therefore compared FA distribution among S-T, M-T, and L-T *Bombus*. The bees were clearly separated where LD1 and LD2 accounted for 72.6% and 27.4% of discriminative power, respectively. L-T and S-T bees were characterized by high levels of MUFAs and M-T bees by high levels of SFAs (Fig. 8 a & b).

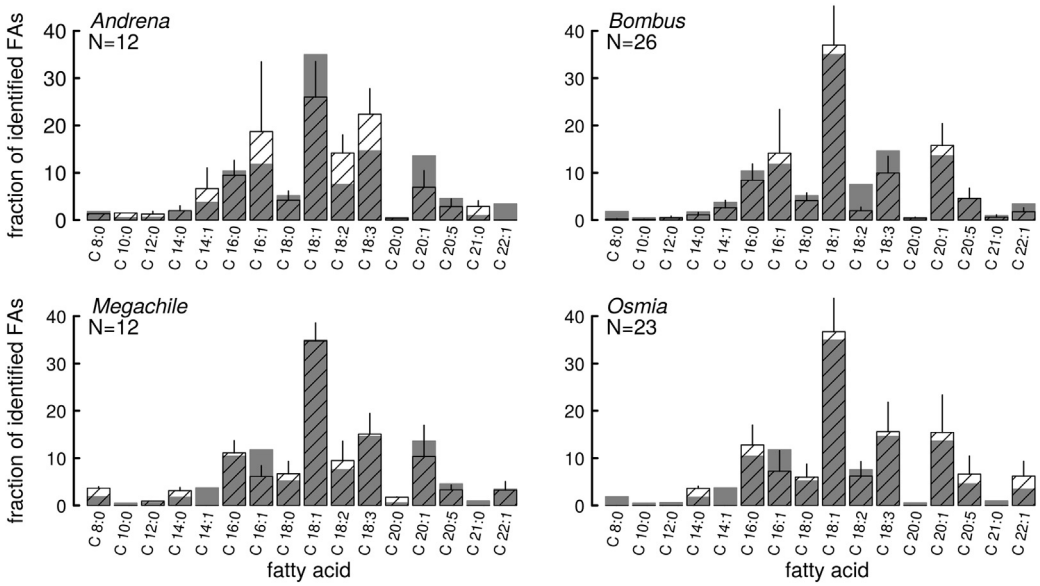

Fig. 5. Percentage contribution of individual fatty acids in the total fatty acids identified. Gray shaded bars represent the percent of each fatty acid (as shown on the x-axis) in all bees combined and the super-imposed hatched bars represent mean ± sd for the genus indicated.

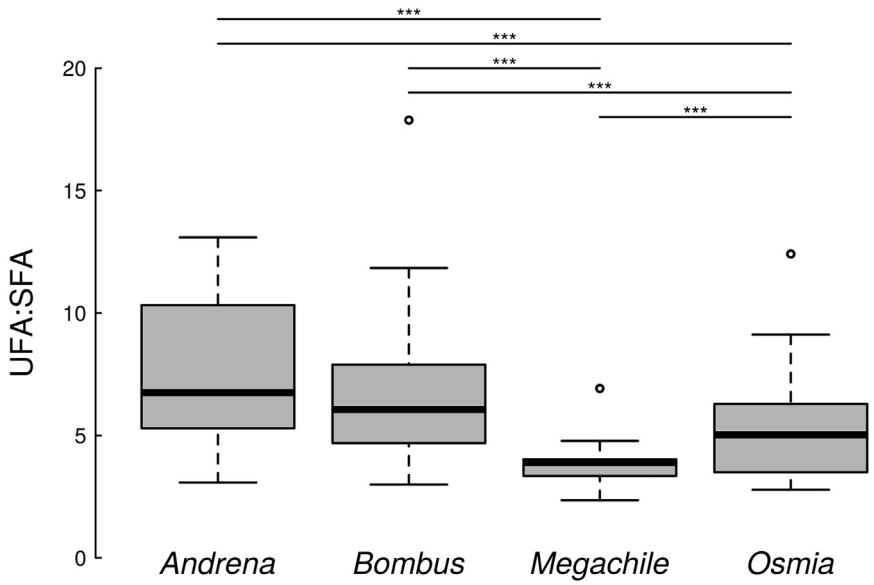

Fig. 6. Boxplot comparing the ratio of UFA:SFA among genera. Significant pairwise comparisons are shown by black bars (***$P \leq 0.001$; see text for statistical details).

3.3. Cluster analysis

The differences among bee genera in FA profile (Figs. 7 & 8) prompted cluster analysis on the fractional contribution of individual FA to the total FAs identified per individual bee to determine the extent to which FA profile can be correlated with the phylogeny of the bees. Fig. 9 shows the results of cluster analysis for the data sets. The aggregations by genus were not completely separated, suggesting potential overlap in dietary acquisition of FAs.

4. Discussion

Among the three methods tested for FAME preparation for FA analysis in bees, we found direct transesterification with KOH to be best suited for samples containing as low as 0.004 g dry mass. Hence, this method of FAME preparation was suitable for the smallest bee analyzed. Further, the same approach may be applicable in even smaller samples if methods to concentrate the extract are employed.

To our knowledge, this study is the first to report FAs with chain-lengths shorter than C 12 in bees. Fatty acids between C 12 to C 20 were reported for worker honey bees (Haddad et al., 2007; Robinson and Nation, 1970) or from C 12 to C 22 in male bumble bees (Cvačka et al., 2006). Fatty acids up to C 24 for either larvae or queen honey bees or some unusual FAs with longer chains, such as C 24, C 26 and C 28 in triacylglycerols isolated from fat tissue of early-nesting male *B. partorum* have been reported (Cvačka et al., 2008). This variation in types of FAs identified in bees from our study versus other studies may be due to the methodological differences to some degree, and may also be an indication of the sensitivity of the method we employed. However, further studies are required to understand whether the differences are due to the species effect or the methodological differences.

Fatty acid composition clearly varied among *Andrena, Bombus, Megachile* and *Osmia*. These differences may be attributed to the variations in their thermal and feeding ecology. Bee genera have different abilities of endothermic heat generation and retention. Thermal ecology of bees is partly determined by three different factors: body

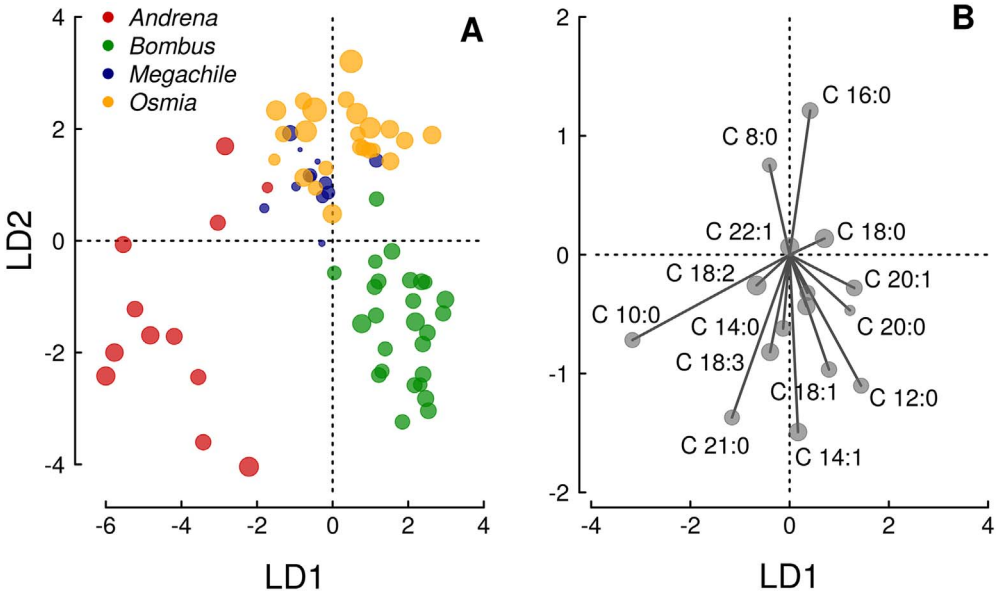

Fig. 7. Linear discriminant analysis of fatty acid composition among bee genera. The first (LD1), second (LD2), and third (LD3) discriminant axes (represented by point size) had discrimination powers of 55.6%, 32.8%, and 11.6%, respectively. (a) Points represent projection values for each individual bee sample, with colors indicating genus (b) Biplot for projection of individual fatty acids along the three discriminant axes as in (a).

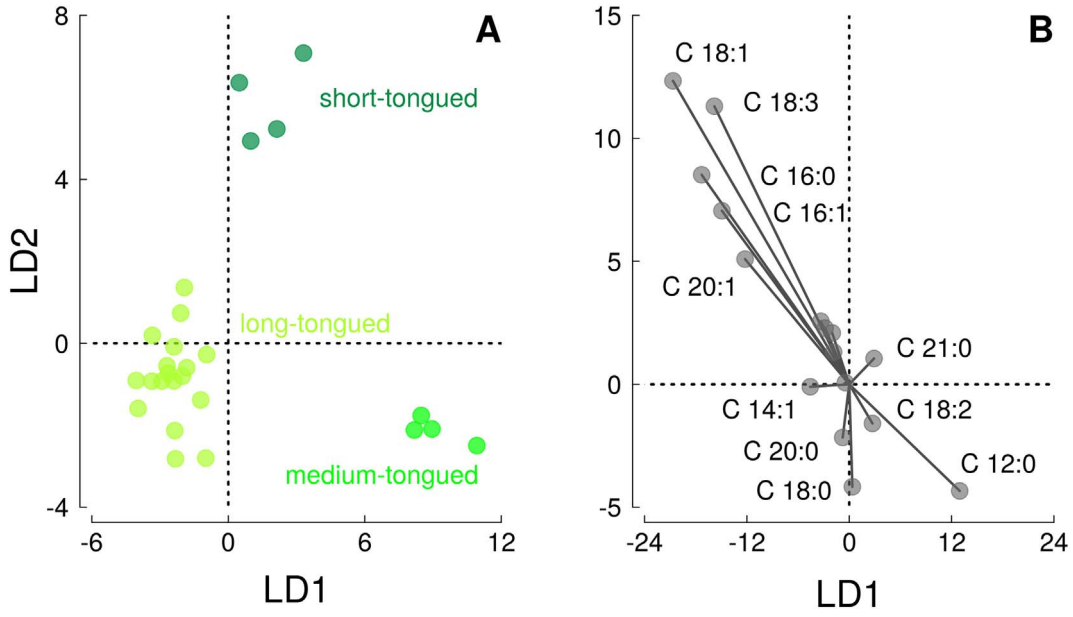

Fig. 8. Projection of samples across the first two discriminants LD1 and LD2 for *Bombus* with different proboscis lengths. (a) Points represent projection values for each bee, with colors representing proboscis length. (b) Biplot for projection of fatty acids along the two discriminant axes as in (a).

size, nesting behavior and the pilosity (both pile length and density). During foraging, heat loss mainly occurs through convection, which is higher for organisms with high surface area: volume ratio (Stone and Willmer, 1989). Hence, smaller bees are more likely to lose heat faster. As the largest bees among the studied genera, *Bombus* are likely to better maintain higher body temperature during foraging given their lower surface area to volume ratio (Bishop and Armbruster, 1999; Stone and Willmer, 1989) and higher pilosity (Nixon and Hines, 2017). Further, because *Bombus* nests in groups, compared to solitary nesting genera, *Bombus* should experience higher and relatively more consistent hive temperatures. However, in contrast to the prediction we made based on the HVA hypothesis, the ratio of UFA:SFA was higher in *Bombus* than in *Osmia* and *Megachile* (Fig. 6). Alternatively, the high UFA: SFA in *Bombus* may be explained by the fact that because, unlike the other three solitary bee genera in this study, *Bombus* is a social bee and may have to produce higher metabolic heat to maintain appropriate nest temperatures and fly at low environmental temperatures. As such, higher levels of UFAs may aid metabolism of lipids and heat production in this group of bees even at low environmental temperatures. Studies in controlled laboratory conditions will be crucial to test this idea. *Andrena,* because of their small body size and solitary nesting behavior, are less able to regulate body temperatures and therefore may have had high UFA: SFA ratio. *Megachile*, on the other hand, which is a solitary nester, had a low UFA:SFA ratio overall but did follow the pattern predicted by surface area to volume ratio i.e., *Megachile*, with larger body size, had significantly lower UFA: SFA than did *Osmia*, which was also the smallest among the bees studied.

The feeding ecology of *Andrena, Bombus, Megachile* and *Osmia* vary with respect to their morphological characteristics and behavioral adaptations (Brian, 1947; Harder, 1985; Inouye, 1980). Acquisition of floral resources in bees is in part based on proboscis length. Bees can be classified as S-T, M-T, and L-T based on proboscis length (Michener et al., 1994), and vary widely in their interactions with flowers, exploiting more diverse floral morphologies than other pollinator taxa

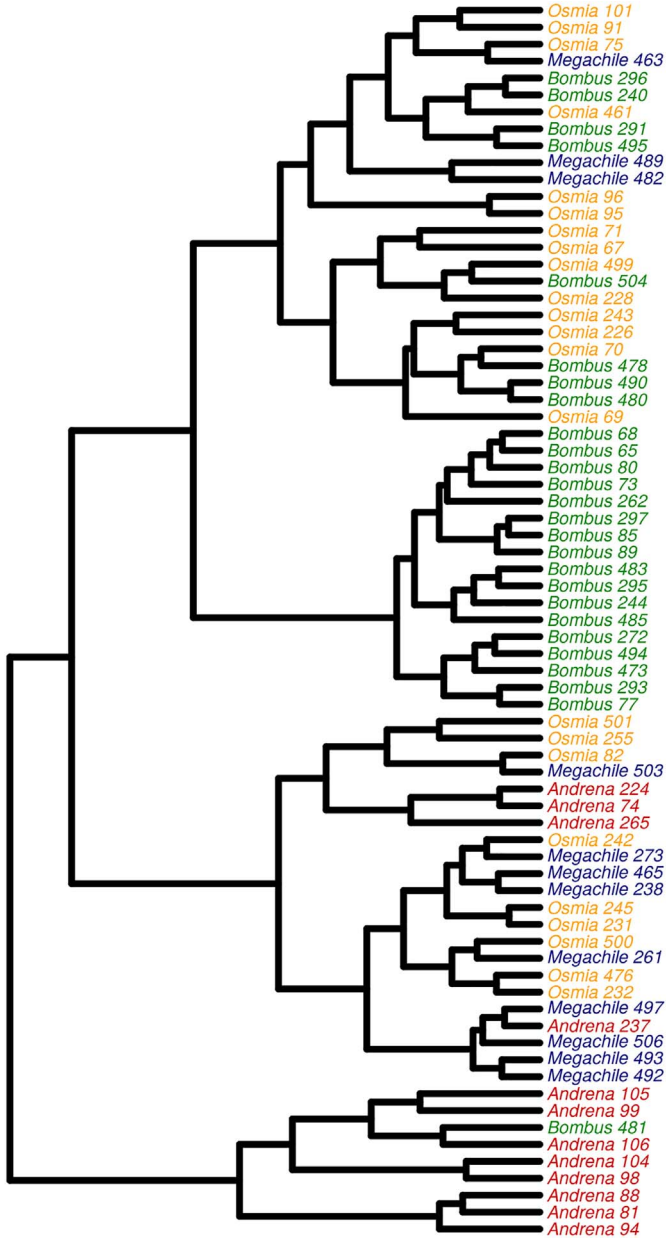

Fig. 9. Hierarchical cluster dendrogram of fatty acid composition in bees obtained from cluster analysis. Individuals with similar FA composition cluster together. Colors as in Fig. 7.

(Willmer, 2011). *Andrena*, as a S-T bee, can collect nectar from open shallow corollas and less so from longer corollas at certain times of the day (excpet when the corollas are full). However, based on their size, *Andrena* may also crawl into the corolla to collect nectar or pollen. As S-T bees, *Andrena* also can forage over a wider range of flowers (Michener et al., 1994; Willmer, 2011). *Bombus, Megachile* and *Osmia* on the other hand, are L-T and specialize in exploiting flowers with deep corollas (Michener et al., 1994; Willmer, 2011). Given that L-T bees can harvest nectar until the last drop, they can have access to it over a much longer period in a day (Willmer, 2011). Within their classification as L-T bees, *Bombus* may again be classified as either S-T bees that lie towards the lower margin, M-T, which lie in the middle or L-T bees, which lie at the higher margin of being described as L-T bees. Our study included 70% L-T, 15% M-T, and 15% S-T *Bombus* (classified based on Koch et al., 2012; Williams et al., 2014). The *Bombus* spp. used in this study can therefore collect nectar or pollen from a wide variety of flowers compared to *Megachile* and *Osmia* likely resulting in more FA types detected. Because *Bombus* are similar in their thermal ecology, differences observed in variables among the different classes based on their proboscis lengths may well represent the effect of dietary FAs on tissue FA composition. Additionally, bees that are endothermic may produce sufficient heat for foraging at relatively colder temperatures and hence weather may affect foraging less. As such, bumble bees may have access to pollen over a wider time window than bees that are less endothermic (Willmer, 2011) and may therefore vary in their consumption of dietary FAs.

Supporting observations from previous studies in insects (Barlow, 1964; Cvačka et al., 2006, 2008; Fast, 1966; Gołębiowski et al., 2012; Robinson and Nation, 1970), C 16:0 and C 18:3 were the most prevalent FAs and C 18:1 was the predominant FA in bees. C 18:3 and a few other PUFAs are considered essential for most insects (Stanley-Samuelson et al., 1988) and hence must be acquired through the diet. The presence of either or both of these FAs in the pollen consumed by the study bees may have led to their 100% prevalence in bees (Human and Nicolson, 2006; Manning, 2006; Markowicz Bastos et al., 2004). Supporting this statement, FA analysis in pollen (Copoco's Honey, CO, USA; $N = 20$) obtained for feeding lab-reared bees showed C 16:0 and C 18:3 to be the most prevalent and predominant (our unpublished data). Another possibility for presence of C 16:0 may be due to the ability of bees to synthesize this FA from other FAs either by the process of chain-elongation, saturation, or even chain-shortening (Stanley-Samuelson et al., 1988) for e.g. C 18:0 to C 16:0 (also see Howard and Blomquist, 2005) where C 18:0 may be obtained from the diet. On the other hand, the process of desaturation and chain elongation in insects is likely similar to that of vertebrates (Stanley-Samuelson et al., 1988). Therefore, by analogy to the vertebrates, C 18:1 may be an intermediate or final step during desaturation and chain elongation of both C 16:0 and C 18:0 (see Fig. 2 in Miyazaki and Ntambi, 2008). Among the two main phylogenetic groups of desaturases, the first group, soluble acyl-acyl carrier protein (ACP) desaturases (found in the plastids of higher plants), is responsible for conversion of SFAs (that are bound to acyl carrier protein) to MUFAs, e.g. for synthesis of C 18:1 (Kachroo et al., 2007). The second desaturase group is made up of i. membrane-bound acyl-lipid desaturases that introduce unsaturated bonds in SFAs in lipid-bound form in plants and cyanobacteria and ii. membrane-bound acyl-coenzyme A (CoA) desaturases that introduce unsaturated bonds in FAs that are bound to acyl-CoA in yeast, fungal and animal cells (Los and Murata, 1998). The latter group of desaturases can be divided into four subfamilies of which the First Desaturases (primarily Δ9 and Δ11 desaturases) are responsible for introducing the first double bond at the 9th or 11th position of the saturated acyl chain (Hashimoto et al., 2008). This subfamily of desaturases have been reported in many groups of insects (Hashimoto et al., 2008; Helmkampf et al., 2015; Howard and Blomquist, 2005), including bees (Buček et al., 2013; Falcón et al., 2014; Lanne et al., 1987), and so could explain the predominance of C 18:1 in bees in this study.

Aside from thermal and feeding ecology, the observed variation in FA composition among bee genera may additionally be the result of varying abilities of the bees to convert one FA type to another. The bee genera studied may vary in their gut microbiota (e.g. Koch and Schmid-Hempel, 2011; Martinson et al., 2011, 2012), which may influence the production of short-chain FAs, the conversion of one FA to another, or FA biosynthesis (e.g. Odelson and Breznak, 1983; Sampedro et al., 2006) as in ruminants (Harfoot and Hazlewood, 1997; Mosley et al., 2002). However, further studies are required to test this idea.

Linear discriminant analysis showed a clear separation of bee genera based on FA composition. It is interesting to see the separation of discriminants of *Andrena*, which is classified as S-T among bee genera from the other three genera that are more closely related to each other (classified as L-T; Figs. 7–9, see the phylogenetic tree from Cardinal and Danforth, 2013). Following a similar argument, *Bombus* was also clearly separated from the Megachilids that are more closely related to each other (see Gonzalez et al., 2012). These differences in FA profile among

these bee genera may be the consequence of their inherent thermal and feeding ecology and are unlikely to reflect differences in intrinsic physiology given that these are usually conserved among distantly-related organisms.

To our knowledge, this study is the first to compare FAs in bees other than commonly recognized honey bees and bumble bees. Our study showed significant differences in FA composition among four bee genera and suggests that the FA composition in organisms may be a reflection of their thermal and feeding ecologies, which may also in part, be used to distinguish among various bee genera. Our results are based on FA composition in whole body tissues. Comparing FA composition among the various tissues within an individual may enhance evaluation at the molecular level. Because bee body temperature may not be exactly similar to environmental temperatures, measuring operative temperatures should also facilitate better comparisons of FA composition and thermal ecology. Comparing FAs among bee tissues and in flowers that individual bees are observed to visit in the field or in diet treatments in laboratory may help determine the link between dietary FAs and tissues FAs in bees.

In conclusion, we report a combination of 16 total FAs in *Andrena, Bombus, Megachile* and *Osmia* with a first published report on presence of FAs with chain-lengths shorter than C 12 in bees. We conclude that FA composition of individuals can be useful in identification of a genus to some degree. We also deduce that thermal and feeding ecology play a role in determining FA composition of bees.

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